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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : A61K 35/52, G01N 33/483, 21/64

A1

(11) International Publication Number: WO 90/13303

(43) International Publication Date: 15 November 1990 (15.11.90)

(21) International Application Number: PCT/US90/02324

(22) International Filing Date: 30 April 1990 (30.04.90)

(30) Priority data:
349,669 10 May 1989 (10.05.89) US

(71) Applicant: THE UNITED STATES OF AMERICA, represented by THE SECRETARY, UNITED STATES DEPARTMENT OF COMMERCE [US/US]; Washington, DC 20231 (US).

(72) Inventor: JOHNSON, Lawrence, A. ; 12516 O'Fallon Street, Silver Spring, MD 20904 (US).

(74) Agents: OLIFF, James, A. et al.; Oliff & Berridge, 277 S. Washington Street, Alexandria, VA 22314 (US).

(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

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Published
With international search report.

(54) Title: METHOD TO PRESELECT THE SEX OF OFFSPRING

(57) Abstract

Intact X and Y chromosome bearing sperm populations of rabbits and swine were separated according to DNA content using a flow cytometer/cell sorter. Sperm viability was maintained by special staining techniques and by sorting and collecting the sperm in nutrient media. The sorted sperm were surgically inseminated into the uteri of rabbits or swine. Of the offspring born from does inseminated with the sorted population of X-bearing sperm, 94 % were females. Of offspring born from does inseminated with sorted Y-bearing sperm from the same ejaculate, 81 % were males.

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METHOD TO PRESELECT THE SEX OF OFFSPRING

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to a method of preselecting the sex of offspring by sorting sperm into X and Y chromosome-bearing sperm based on differences in DNA content.

Description of the Prior Art

Gender of animal offspring is important to livestock producers. Because the dairy farmer has little use for most bull calves, the use of sexed semen to produce only females would make mild production more efficient. Swine farmers would produce pork more efficiently if they were able to market only female swine, because females grow faster than males.

In beef cattle and sheep breeds, the male grows at a faster rate than the female and hence is preferred for meat production.

In addition, the ability to specify male or female offspring should shorten the time required for genetic improvements, since desirable traits are often associated with one or the other parent. Planning the sex of cattle offspring is already practiced on a limited basis. This procedure consists of removing embryos from the cow, identifying their potential gender, and reimplanting only those of the desired gender. However, an ability to separate sperm into male-producing and female-producing groups before they are used for artificial insemination could enhance the overall value of offspring produced by embryo transfer.

Every living being has a set of paired chromosomes, which carry all the genetic material necessary to maintain life and also to propagate new life.

All but one pair of chromosomes are called autosomes and carry genes for all the characteristics of the body, such as skin, hair and eye color, mature size, and body characteristics. The remaining pair are called sex chromosomes. They carry the genetic material that specifies gender. One sex chromosome is called X, the other Y.

A sperm from the male or an egg from the female contains one of each pair of autosomes; in addition, in mammals the egg always contains an X chromosome, while the sperm always carries either an X or Y chromosome.

When a sperm and egg unite and the sperm carries the Y chromosome, the offspring is male (XY); however, if the sperm carries an X chromosome when it unites with the egg, the resulting offspring is female (XX).

The only established and measurable difference between X and Y sperm that is known and has been proved to be scientifically valid is their difference in deoxyribonucleic acid (DNA) content. The X chromosome is larger and contains slightly more DNA than does the Y chromosome. The difference in total DNA between X-bearing sperm and Y-bearing sperm is 3.4% in boar, 3.8% in bull, and 4.2% in ram sperm.

The amount of DNA in a sperm cell, as in most normal body cells, is stable. Therefore, the DNA content of individual sperm can be monitored and used to differentiate X- and Y-bearing sperm.

Since the difference in DNA mass in the sex chromosomes of most mammals is the only scientifically validated, measurable difference between X- and Y-bearing sperm, the chromosomal constitution [Moruzzi, J. Reprod. Fertile. 57: 319 (1979)] and/or measurement of DNA mass [Pinkel et al (1), Science 218: 904 (1982); Pinkel et al (2), Cytometry 3: 1 (1982); Johnson and Pinkel, Cytometry 7: 268 (1986); Johnson et al (1), Gam. Res. 16: 1 (1987); Johnson et al (2), Gam. Res. 17: 203 (1987)] are the only verifiable means other than fertility for determining sex-producing capability of a population of sperm. The literature describes many physical, biochemical, and functional methods that have purportedly sexed sperm [Amann and Seidel, "Prospects for Sexing Mammalian Sperm," Colorado Assoc. Univ. Press, Boulder (1982)]; several of these methods have been tested for relative

DNA content [Pinkel et al., J. Anim. Sci. 60: 1303 (1985); Johnson (1), Theriogenology 29: 265 (1988)]. However, no method has been proven in controlled experiments to actually affect the sex ratio of offspring.

Previous studies have demonstrated that the difference in DNA content between X and Y chromosome-bearing sperm can be repeatedly measured and the sperm sex ratio of a sample of semen predicted [Johnson and Pinkel, supra; Johnson et al (1), supra; Johnson et al (2), supra; Johnson (1), supra; Johnson (2), Cytometry, Suppl. 2: 66 (Abstract) (1988)]. Verifiable separation by sorting of X and Y sperm based on DNA content has been accomplished with the vole [Pinkel et al (1), supra; Johnson, In "Beltsville Symposia in Agricultural Research X," P.C. Augustine, H.D. Danforth, & M.R. Bakst (eds.), Martinus Nijhoff, Boston, pp. 121-134 (1986)] and the chinchilla (Johnson et al (1), supra]. However, preparation procedures damaged DNA viability. The sorting of sperm nuclei from several mammalian (bull, boar, ram, vole, chinchilla) species into separate X and Y chromosome-bearing populations at purities ranging from 92 to 99% has been accomplished [Johnson and Clarke, Gam. Res. 21: 335 (1988)]. Nuclear decondensation and pronuclear development was demonstrated in hamster eggs that had been microinjected with sorted X- or Y-bearing bull, boar, or ram sperm [Johnson and Clarke, supra].

SUMMARY OF THE INVENTION

It is an object of this invention to provide a method for sorting mammalian sperm into X and Y chromosome fractions based on DNA content.

It is a further object of this invention to teach a method of staining the DNA of mammalian sperm while maintaining viability of the sperm.

It is a further object of this invention to provide a sheath fluid adapted to be used in a cell-sorting apparatus while maintaining viability of sperm cells.

It is a further object of this invention to provide a collecting fluid capable of maintaining the viability of sorted sperm cells.

Other objects and advantages of this invention will become readily apparent from the ensuing description.

DETAILED DESCRIPTION OF THE INVENTION

I have now demonstrated the separation, by flow sorting, of intact, viable X and Y chromosome-bearing rabbit and swine sperm populations based on relative DNA content; surgical insemination of the sorted sperm into does; and the subsequent birth of sexed offspring with a phenotypic sex ratio consistent with predictions based on the relative DNA content of the sorted sperm populations.

A flow cytometer measures the amount of fluorescent light given off when the sperm, previously treated with a fluorescent dye, pass through a laser beam. The dye binds to the DNA. The fluorescent light is collected by an optical lens assembly; the signal is transported to a photomultiple tube, amplied, and analyzed by computer. Because the X chromosome contains more DNA than the Y chromosome, the female sperm (X) takes up more dye and gives off more fluorescent light than the male sperm (Y).

For small differences in DNA to be detected between X and Y, the sperm must pass single file through the laser beam, which measures the DNA content of individual sperm.

In orthogonal flow cytometry, a suspension of single cells stained with a fluorochrome is made to flow in a narrow stream intersecting an excitation source (laser beam). As single cells pass through the beam, optical detectors collect the emitted light, convert the light to electrical signals, and the electrical signals are analyzed by a multichannel analyzer. The data are displayed as multi-or single-parameter histograms, using number of cells and fluorescence per cell as the

coordinates.

In order to use an orthogonal flow cytometric system to differentiate between X- and Y-bearing sperm DNA, a beveled sample injection tip and a second fluorescence detector in the forward position is required [Johnson and Pinkel, supra]. This paper is herein incorporated by reference. The modified system allows one to control the orientation of the flat ovoid sperm head as it passes the laser beam. Elimination of the unoriented sperm by electronic gating enhances precision. Typically, 80% of sperm nuclei (without tails) are properly oriented as they pass the laser beam.

In the modified Epics V flow cytometer/cell sorter, hydrodynamic forces exerted on the flat, ovoid mammalian sperm nuclei orient the nuclei in the plane of the sample stream as they exit the beveled injection tip. Fluorescent signals are collect simultaneously by 90 and 0 degree optical detectors, from the edge and flat side of the sperm nucleus, respectively. For sorting, the sample stream is broken into uniform droplets by an ultrasonic transducer. Droplets containing single sperm of the appropriate fluorescence intensity are given a charge and electrostatically deflected into collection vessels. The collected sperm nuclei then can be used for microinjection into eggs. Since the sperm nuclei have no tails, they cannot be used for normal insemination.

Accurate measurement of mammalian sperm DNA content using flow cytometry and cell sorting is difficult because the sperm nucleus is highly condensed and flat in shape, which makes stoichiometric staining difficult and causes stained nuclei to have a high index of refraction. These factors contribute to emission of fluorescence preferentially from the edge or thin plane of the sperm nucleus. In most flow cytometers and sorters, the direction of sample flow is orthogonal to the direction of propagation of the laser beam and the optical axes of the fluorescence detection. Consequently,

fluorescence measurement is most accurate when the sperm fluorescence is excited and measured on an axis perpendicular to the plane of the sperm head [Pinkel et al (2), supra]. At relatively low sample flow rates, hydrodynamics are used to orient tailless sperm so that DNA content can be measured precisely on 60 to 80% of the sperm passing in front of the laser beam. The modified Epics V system used in this study can measure the DNA content of tailless sperm from most species at the rate of 50 to 150 sperm per second [Johnson and Pinkel, supra].

Intact sperm (with tails), whether viable or nonviable, cannot be oriented as effectively as tailless sperm nuclei [Johnson (2), supra]. However, a 90-degree detector can be used to select the population of properly oriented intact sperm to be measured by the 0 degree detector. Since no hydrodynamic orientation is attempted, the sample flow rate can be much higher, which compensates somewhat for the fact that only 15 to 20% of intact sperm pass through the laser beam with proper orientation. In this invention, the overall flow rate was approximately 2500 intact sperm per second. The intact X-and Y-bearing sperm fractions were sorted simultaneously from the population of input sperm at a rate of 80-90 sperm of each type per second.

It is, of course, of critical importance to maintain high viability of the intact sperm during the sorting process and during storage after sorting but prior to insemination.

Of the factors involved in maintaining sperm viability, the method of staining the sheath fluid, and the collecting fluid have been found to be especially important.

A nontoxic DNA stain must be selected. A preferred stain is Hoeschst bisbenzimide H 33342 fluorochrome (Calbiochem-Behring Co., La Jolla, CA). To our knowledge, this fluorochrome is the only DNA binding dye

that is nontoxic to sperm. Concentration of the fluorochrome must be minimal to avoid toxicity, and yet be sufficient to stain sperm uniformly and to detect the small differences in the DNA of X and Y sperm with minimal variation. A suitable concentration was found to be 5 μg/ml, but this may be varied from 4 to 5 μg/ml.

The sperm must be incubated with stain at sufficient temperature and time for staining to take place, but under mild enough conditions to preserve viability. Incubation for 1 hour at 35°C would also be effective. Incubation time has to be adjusted according to temperature; that is, 1.5 hr. for 30°C; 1 hr for 39°C.

Sheath fluid used in sorting cells must be electrically conductive and isotonic. A concentration of 10 mM phosphate buffered saline provided the necessary electrical properties, and 0.1% bovine serum albumin was added to enhance sperm viability by providing protein support for metabolism and viscosity for the sperm. The sheath fluid must be free of sugars and excess salts.

Dilution of sperm as occurs in sorting tends to reduce viability of the cells. To overcome this problem sperm were collected in test egg yolk extender [Graham et al, J. Dairy Sci. 55: 372 (1972)] modified by adjusting the pH and adding a surfactant. Details of the composition of the extender are shown in Example 1. The surfactant is believed to enhance capacitation of the sperm prior to fertilization.

To confirm the DNA content and predict the sex of the offspring of surgically inseminated X or Y sorted sperm fractions, an aliquot of the sorted sperm was sonicated to remove the tails, stained, and the nuclei was reanalyzed for DNA content to predict the proportion of X and Y sperm.

Although the detailed description which follows uses the sorting of rabbit sperm as an example of this invention, it is expected that the sperm of most mammals could be effectively sorted by following these proce-

dures. Those skilled in the art will recognize that minor modifications may be made in the procedure without departing from the spirit and scope of the invention.

Rabbit semen was collected, diluted, and stained with a fluorochrome dye. Sperm were sorted in a modified Epics V flow cytometer/cell sorter.

After being sorted, sperm were surgically inseminated into the uteri of rabbits.

The results obtained by surgical insemination of does with sorted intact sperm are presented in Table I. Recovery of ova 40 hr post-insemination indicated that stained sorted sperm, as well as unstained unsorted sperm, were capable of fertilizing rabbit ova <u>in vitro</u>.

Table I. Fertilizing Capacity of Flow-Sorted Rabbit Spermatozoa After Intrauterine Insemination of Does

	Number of			
Treatment of Sperm	Does Inseminated	Ovulation Points	Eggs Recovered	Eggs Fertilized
Unsorted	2	16	9	9
Sorted	6*	59	46	39

* One doe accounted for 7 recovered and 7 unfertilized eggs.

Table II. Predicted and Actual Sex Ratios of Offspring After Intrauterine Insemination of Sorted X and Y Chromosome-bearing Rabbit Sperm

Treatment of Sperm	Number of Does		Total No. of Young Born	Percentage and Numbers of Offspring			
				Predicted		Actual	
	Inseminated	Kindling		% Males	% Females	% Males (N)	% Females (N)
Sorted Y	16	5	21	81	19	81 (17)	19 (4)
Sorted X	14	3	16	14	86	6 (1)	94 (15)
Recombined X and Y	17	5	14	50	50	43 (6)	57 (8)
Total	47	13	51	--	--	47 (24)	53 (27)

Inseminations were also made to determine the comparability of predicted sex of offspring to phenotypic sex. As the data in Table II indicate, the predictability of the phenotypic sex based on DNA analysis of the separated intact sperm was very high. Reanalysis of the sorted Y population used for insemination indicated that 81% of the sperm were Y-bearing. The sex ratio of offspring from these inseminations was identical to that predicted. These values were significantly different from theoretical 50:50 sex rates (P<.003). Reanalysis of the sorted X-bearing sperm population used for insemination indicated that 86% were X-bearing and 14% were Y-bearing sperm. The phenotypic sex of the offspring from these inseminations was 94% female, which was different from the theoretical 50:50 (P<.0003).

Inseminations were made with sorted X and Y populations that were recombined (recombined X and Y group) immediately before insemination. The assumption was made that the proportions of X and Y in the recombined samples were equal (50:50). The phenotypic sex resulting from the inseminations was 57% female and 43% male (Table II) and was not significantly different from the theoretical (50:50) sex ratio (P = .40). The phenotypic sex ratio of offspring born of does inseminated with either sorted X-bearing or sorted Y-bearing sperm was different (P<.0002 for X and P<.001 for Y) from the theoretical (50:50) sex ratio expected from untreated semen.

Embryonic mortality was significant in the does inseminated with sorted intact sperm. With a reasonably high fertilization rate (Table I), one would expect a kindling rate of near 80% and litter size of about six from does of this age and breed. However, the kindling rate across the three treatment groups averaged 28%, with an average litter size of 3.9. The cause of the apparent high rate of embryonic death is thought to be due to the fluorochrome binding to the DNA and/or to the effect of

the laser beam exciting the DNA bound fluorochrome. Earlier work has shown that sorted vole sperm nuclei that were microinjected into hamster eggs exhibited chromosome breakage in the developing sperm pronucleus [Libbus et al, Mut. Res. 182: 265 (1987)]. Those sperm had been sonicated, stained, sorted, and microinjected, a somewhat more rigorous treatment than the staining and sorting used in this study.

I have demonstrated that DNA can be used as a differentiating marker between X- and Y-bearing sperm, that DNA can be used to accurately predict the sex of offspring from separated X- and Y-bearing sperm populations, and that flow sorting is an effective means for separating viable X- and Y-bearing sperm populations suitable for production of offspring.

The following examples are intended only to further illustrate the invention and are not intended to limit the scope of the invention, which is defined by the claims.

Example 1

Semen was collected from mixed breed mature bucks by use of an artificial vagina. Sperm concentration was determined with a hemocytometer. The semen was diluted with Tris buffer, pH 6.9, to a concentration of 10×10^6 per ml. Bisbenzimide H 33342 fluorochrome was added at a concentration of 5 μg/ml. The samples were incubated for 1 hr at 35°C. Intact sperm were sorted on a modified EPICS V flow cytometer/cell sorter. The stained intact sperm were excited in the ultraviolet (UV; 361 and 364 nm) lines of a 5-watt 90-5 Innova Argon-ion laser operating at 2000mW. Data were collected as 256-channel histograms. Sheath fluid was 10 mM phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). Sperm were sorted into a test egg yolk extender.

The composition of the extender was N-tris(hydroxymethyl)-methyl-2-amino ethane sulfonic acid, 2.16 g;

tris hydroxymethyl aminomethane, 0.51 g; dextrose, 0.1 g; streptomycin sulfate, 0.13 g; penicillin G, 0.08 g; egg yolk, 12.5 ml; Equex STM (Nova Chemical Sales, Scituato, MA), 0.5%; and distilled water, 50 ml. This mixture was centrifuged, and only the supernatant was used. The sorted sperm were concentrated by incubating at room temperature for 1 hr, after which the more dilute fraction was removed and the remainder was used for insemination 1 to 4 hr later.

Example 2

Mature New Zealand White does were injected with 150 international units of human chorionic gonadotropin (HOG) to induce ovulation, which was expected to occur 10 hr later. Seven hours after treatment with HOG, the does were surgically prepared by injection with Ketamine hydrochloride containing acepromazine and anesthetized under halothane and oxygen. The uterus was exposed by midline incision, and 100 μl of sorted or unsorted sperm was placed into the lumen of the anterior tip of each uterine horn through a 21-guage needle. Standard management practices were used in caring for the rabbits. These does were sacrificed 40 hr post-insemination; uteri were flushed and recovered eggs evaluated. All fertilized eggs recovered were classified as morula. The results of these experiments are shown in Table I.

Example 3

Table II shows the results of inseminations made into the tip of the uterine horn: the number of does that kindled and the phenotypic sex of the offspring compared to the predicted sex. Predicted sex of offspring was based on reanalysis of sorted intact sperm to determine relative DNA content. For reanalysis, the sorted sperm was sonicated for 10 sec and centrifuged at 15,000 g, the supernatant was discarded, and the pellet was resuspended in 9 μM bisbenzimide H 33342. Phenotypic sex of the offspring was determined soon after birth and

confirmed at later ages up to 10 weeks. Recombined X and Y is the sorted X and Y sperm populations recombined immediately before insemination.

Example 4

Using the methods of Examples 1, 2, and 3, viable swine sperm was sorted into viable X and Y chromosome-bearing populations. Two litters (18 pigs) from surgically inseminated boar semen produced 88% females from X-sorted sperm and 67% males from Y-sorted sperm.

It is understood that the foregoing detailed description is given mainly by way of illustration and that modification and variation may be made therein without departure from the spirit and scope of the invention.

WHAT IS CLAIMED IS:

1. A method to preselect the sex of mammalian offspring comprising:

a) collecting sperm from a male mammal;

b) staining said sperm with a fluorescent dye capable of selectively staining DNA in living cells;

c) diluting said sperm with a viability supporting sheath fluid;

d) passing said sheath fluid containing said sperm through detecting means for said stained DNA and a cell sorting means;

e) selecting by said cell sorting means said sperm which will produce the desired gender of off-spring;

f) collecting said selected sperm in a viability-supporting collecting fluid;

g) inseminating a female mammal of the same species as said male mammal with said sorted sperm in said collecting fluid.

2. The method of Claim 1 wherein said mammal is a rabbit.

3. The method of Claim 1 wherein said mammal is a swine.

4. The method of Claim 1 wherein said dye is bisbenzimide H 33342 fluorochrome.

5. The method of Claim 1 wherein said sheath fluid is phosphate buffered saline solution containing 0.1% bovine serum albumin.

6. The method of Claim 1 wherein said collecting fluid is modified test egg yolk extender.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/02324

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

U.S. CL: 424/561, 436/63, 172
IPC (5): A61K 35/52, G01N 33/483, G01N 21/64

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	424/561; 436/63, 172

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

CAS, BIOSIS, APS

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	US, A, 4,083,957 (LANG) 11 April 1978, See the abstract and column 1.	1-6
Y	GB, A, 2,145,112 MILK MARKETING BOARD (UNITED KINGDOM), 20 March 1985, See the entire document	1-6
Y	GAMETE RESEARCH, Volume 21, Number 4, issued 29 June 1988, Johnson et al., "Flow Sorting of X and Y Chromosome-Bearing Mammalian Sperm", pages 335-344, See the entire document	1-6
X,P	BIOLOGY OF REPRODUCTION, Volume 41, issued 12 July 1989, Johnson et al., "Sex Preselection in Rabbits: Live Births form X and Y Sperm Separated by DNA and Cell Sorting", See the entire document	1-6

* Special categories of cited documents: [15]

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IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
19 JUNE 1990	14 AUG 1990
International Searching Authority [1]	**Signature of Authorized Officer [20]**
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